Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 560850, 8 pages
doi:10.1155/2011/560850

Research Article

Alpha 1,3-Galactosyltransferase Deficiency in Pigs Increases Sialyltransferase Activities That Potentially Raise Non-Gal Xenoantigenicity

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Received 29 May 2011; Revised 29 July 2011; Accepted 15 August 2011

Academic Editor: Saulius Butenas

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We examined whether deficiency of the GGTA1 gene in pigs altered the expression of several glycosyltransferase genes. Real-time RT-PCR and glycosyltransferase activity showed that 2 sialyltransferases [α2,3-sialyltransferase (α2,3ST) and α2,6-sialyltransferase (α2,6ST)] in the heterozygote GalT KO liver have higher expression levels and activities compared to controls. Enzyme-linked lectin assays indicated that there were also more sialic acid-containing glycoconjugate epitopes in GalT KO livers than in controls. The elevated level of sialic-acid-containing glycoconjugate epitopes was due to the low level of α-Gal in heterozygote GalT KO livers. Furthermore, proteomics analysis showed that heterozygote GalT KO pigs had a higher expression of NAD^+-isocitrate dehydrogenase (IDH), which is related to the CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) enzyme reaction. These findings suggest the deficiency of GGTA1 gene in pigs results in increased production of *N*-glycolylneuraminic acid (Neu5Gc) due to an increase of α2,6-sialyltransferase and a CMAH cofactor, NAD^+-IDH. This indicates that Neu5Gc may be a critical xenoantigen. The deletion of the CMAH gene in the GalT KO background is expected to further prolong xenograft survival.

1. Introduction

The pig is the best candidate species for clinical transplantation into humans. However, the α-Gal epitope is a major obstacle to successful xenotransplantation [1]. The enzyme α1,3-galactosyltransferase (GalT) catalyzes the binding of α1,3galactose (Gal) on *N*-acetyllactosamine (Galβ1,4GlcNAc) to produce Galα1,3Galβ1,4GlcNAc-R (α-Gal epitopes) on the cell surface of almost all mammals, but not on those of humans, apes, and Old World monkeys [2]. Several research groups have produced α1,3-galactosyltransferase (GGTA1) gene knockout (GalT KO) pigs in order to overcome the problem of immune rejection after xenotransplantation [3–6]. Organs from these pigs avoid both hyperacute and acute humoral xenograft rejection without requiring complement inhibition or antibody absorption [7].

Although GalT KO-derived organs prolong xenograft survival in recipients, xenografted organs from these animals result in progressive organ death [8]. Carbohydrates such as Hanganutziu-Deicher (H-D), Thomsen-Friedenreich (T or TF), Tn, and sialyl-Tn play a pivotal role in the acute immune rejection of pig xenografts [9]. H-D antigens are glycoconjugate-bound *N*-glycolylneuraminic acids (Neu5Gc) which are a type of sialic acid (Sia), as is *N*-acetylneuraminic acid (Neu5Ac). In cells, Neu5Gc is mainly produced from Neu5Ac by the catalyst CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) with the cofactors cytochrome b_5 and NADH [10, 11]. In this study, we tested whether Sia-containing glycoconjugate expression in pigs could be altered by deficiency of the GGTA1 gene. To accomplish this, we examined whether increased production of Neu5Gc seen in heterozygote GalT KO pigs was caused by

increased expression of α2,6-sialyltransferase (α2,6ST) and the CMAH cofactor NAD^+-isocitrate dehydrogenase (IDH).

2. Material and Methods

2.1. Sample Preparation and Protein Determination. In this study, we used 3 control and 3 GalT heterozygote KO pigs ranging in age from 4 to 6 weeks. GalT heterozygote pigs were created as previously reported [6]. The treatment of the pigs used in this research followed the guidelines set by the National Institute of Animal Science's Institutional Animal Care and Use Committee, Suwon, Republic of Korea (approval no. 2009-004, D-grade). Control and heterozygote GalT KO livers were minced with a tissue grinder under liquid nitrogen. The organ powders were washed twice with phosphate buffered saline (PBS) and then centrifuged at 1,500 ×g for 10 min. The pelleted organ powders were resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) containing 0.5% Nonidet P-40, protease inhibitor cocktail (Roche, Almere, Netherlands), and lysed by sonication. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) with a bovine serum albumin standard.

2.2. RNA Isolation and Real-Time RT-PCR. Total RNA was extracted from control and heterozygote GalT KO liver tissue using a Micro-to-Midi total RNA Purification System (Invitrogen, La Jolla, CA, USA). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using a DNA Engine Chromo4 system (Bio-Rad, Hercules, CA, USA) and SYBR Green as the double-stranded DNA-specific fluorescent dye (Bio-Rad, Hercules, CA, USA). We used pig H2A histone family, member Z (pH2AFZ) as an internal standard to normalize the RT-PCR reaction efficiency and to quantify in heterozygote GalT KO pig- and control-derived liver mRNA. After normalization with pH2AFZ mRNA, we compared the relative expression of each mRNA in the heterozygote GalT KO pig-derived liver genes with those of the controls. We performed RT-PCR on each sample independently and in triplicate. Data are presented as the mean of the gene expression measurements for each individual control and heterozygote GalT KO liver sample (Table 1).

2.3. Assay of Glycosyltransferase Activity. The α1,3-galactosyltransferase (GalT), α2,3- and α2,6-sialyltransferase (α2,3ST and α2,6ST) activity were assayed as previously described with minor modifications [12, 13]. In brief, an acceptor substrate, lacto-*N*-neotetraose (LNnT; Sigma-Aldrich) was labeled with 2-aminobenzamide (2-AB). The mixture was comprised of 2 mg of LNnT, 0.2 mg of 2-AB, 0.24 mg of sodium cyanoborohydride, 6 μL of acetic acid, and 14 μL of dimethyl sulfoxide (DMSO). The mixture was incubated at 65°C for 3 h and then was purified using GlycoClean S Cartridges (ProZyme, Hayward, CA, USA). The assay mixture for GalT activity contained 20 mM HEPES buffer (pH 7.2), 0.25% Nonidet P-40, 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl, 20 mM UDP-galactose, 200 mM galactose, and 100 μM acceptor substrate (LNnT-AB), and 6 μL of organ lysate for a total volume of 20 μL. The assay mixture for α2,3ST and α2,6ST activity contained 20 mM HEPES buffer (pH 7.2), 0.25% Nonidet P-40, 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl, 20 mM CMP-Neu5Ac, 200 mM galactose, and 1 mM acceptor substrate (LNnT-AB), and 6 μL of organ lysate for a total volume of 20 μL. After incubation at 37°C for 6 h, 80 μL of water was added to each sample mixture and the reaction was terminated by boiling for 5 min, followed by centrifugation of the samples at 15,000 ×g for 10 min. The resulting supernatant was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) using an octadecyl silane (ODS) column (4.6 × 150 mm, TSK-gel column ODS-80TM; Tosoh Bioscience, Tokyo, Japan). The products and substrate were isocratically separated with 20 mM ammonium acetate buffer (pH 4.0) containing 0.15% *n*-butanol at 55°C. Each peak was detected with a fluorescence detector (Model RF-10A; Shimadzu, Tokyo, Japan) at excitation and emission wavelengths of 330 and 420 nm, respectively. We defined enzyme activity as picomoles of product per hour per milligram of organ lysate protein. Product amounts were determined from fluorescence intensities using 2-aminobenzamidylated LNnT as a standard.

2.4. Enzyme-Linked Lectinosorbent Assays (ELLAs). Control and heterozygote GalT KO livers were also tested by ELLA, using *Griffonia simplicifolia* isolectins B4 (GS-IB4), *Maackia amurensis* agglutinin (MAA), and *Sambucus nigra* agglutinin (SNA). A 50 μL sample of organ lysate (25 μg protein/well) was diluted in PBS, dispensed into 96-well microtiter plates and incubated at room temperature for 2 h. The organ lysates were then dispersed and washed once with PBS containing 0.1% Tween 20 (PBST) and blocked with PBST containing 2% bovine serum albumin. Biotinylated GS-IB4, MAA, and SNA solutions (all 100 μL at 0.1 μg/mL) were applied and incubated at room temperature for 2 h. The samples were washed 3 more times with PBST and incubated for another 2 h with 100 μL of horseradish peroxidase-conjugated hen egg white avidin (0.1 μg/mL). The reaction was developed using o-phenylenediamine dihydrochloride (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Absorbance was measured at 490 nm using a Multiskan FC Microplate Photometer (Thermo Scientific, Pittsburgh, PA, USA) and signal levels were normalized using actin.

2.5. 2-Dimensional Gel Electrophoresis Analysis and Protein Identification. The 2-dimensional gel electrophoresis (2DE) and spot analysis were performed as previously described [14], with slight modifications. Total proteins (500 μg) for analytical runs were transferred into IPG strip holder channels (Bio-Rad, Hercules, CA, USA). The 2DE process separates protein mixtures by IEF (pH 3–10) in the first dimension and SDS-PAGE (7.5–17.5% linear gradient) in the second dimension. The resulting gels provide high-resolution separation of a complex mixture of proteins. Target spots, identified using PDQuest software (Bio-Rad, Hercules, CA, USA), were excised from the gel, destained, and subjected to in-gel digestion with bovine trypsin (Roche, Almere, Netherlands). We created a match set consisting of 6 images, 3 from control and 3 from heterozygote GalT

Table 1: Primer sequences used for real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Genes	Primer sequences (5′–3′)
CMAH full-length	Forward: gaaagaccaagtcgggaaca Reverse: cttccagtcaggtggtgtga
CMAH variant 2	Forward: gagcttcaggctttccaatg Reverse: tccagccatacttgtctgct
CMAH variant 3	Forward: gatggcgttcatcctgaga Reverse: ccaactccttggcatcattt
ST3Gal 1	Forward: gcatcctctccgtgatcttc Reverse: caagatggttgtcacgttgg
ST3Gal 3	Forward: gcttcaagtggcaggacttc Reverse: atgaggccattgttgaaagg
ST3Gal 4	Forward: gccatcaccagctattccat Reverse: gtgggcagattcagggtaga
ST6Gal 1	Forward: tgtgtgaccaggtggatgtt Reverse: tcccaagcaggtagatgtcc
ST6GalNAc 5	Forward: cccgatgaatgcacaatgta Reverse: cctcagaacacgggtttgtt
pH2AFZ	Forward: ggtaaggctgggaaggactc Reverse: gatgcatttcctgccaattc

KO livers. One of the control images was selected as the match set standard for spot matching. We removed the background from each gel image. The protein abundance of detected spots was quantified and normalized by dividing the optical density (OD) values of individual spots by the total OD values of all spots present in the images. Peptides were then analyzed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously [15]. Briefly, trypsin digestion reactions were terminated with trifluoroacetic acid (TFA) at a final concentration of 10%. Peptides were concentrated and desalted using ZipTipm-c18 (Millipore, Etten-Leur, Netherlands) and eluted directly onto the MALDI target in 1 mL of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile. Peptides were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) in reflection mode, at an accelerating voltage of 20 kV. Database searches were performed using Protein Prospector (http://propector.ucsf.edu) and PROWL (http://www.proteometrics.com).

2.6. Statistical Analysis. Values are reported as means ± standard deviation (SD). Real-time RT-PCR in Figure 1, glycosyltransferase activity assay in Figure 2, and ELLA in Table 2 were analyzed using 3 controls and 3 heterozygote GalT KO pigs. Statistical significance was determined using the t-test.

3. Results

The sialyltransferase family is generally classified into 4 different subfamilies, ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia, according to the carbohydrate linkages synthesized [16]. We examined how deficiency of GGTA1 gene changed the sialyltransferase mRNA expression in heterozygote GalT KO liver. We used pH2AFZ as an internal standard to normalize the RT-PCR reaction efficiency and to quantify in heterozygote GalT KO pig- and control-derived liver mRNA. After normalization with pH2AFZ mRNA, the mRNA expression of heterozygote GalT KO pig- and control-derived liver genes showed that ST3Gal 1, ST3Gal 3, and ST6Gal 1 gene expressions were upregulated (1.55-fold, $P < 0.001$; 1.13-fold, $P < 0.05$; 3.16-fold, $P < 0.001$, resp.), whereas ST6GalNAc 5 was downregulated (0.49-fold, $P < 0.001$) in heterozygote GalT KO liver compared to the control (Figure 1). This suggests heterozygote GalT KO pig-derived organs exhibit a higher Sia-containing glycoconjugate on glycoprotein and glycolipid than controls, indicating that they act as an immune antigen in allo- or xeno-grafted organs.

Table 2: Signal intensities of enzyme-linked lectinosorbent assays (ELLA).

Lectin	Control	GalT KO
GS-IB4***	3.3282 ± 0.1775	2.1138 ± 0.1864
MAA*	3.5984 ± 0.1192	5.0459 ± 0.2541
SNA*	20.0019 ± 2.2285	25.8502 ± 1.0457

Each value is the mean ± standard deviation (SD) of triplicate determinations. GS-IB4: *Griffonia simplicifolia* isolectin B4; MAA: *Maackia amurensis* agglutinin; SNA: *Sambucus nigra* agglutinin; $^{*}P < 0.05$, $^{***}P < 0.001$.

As shown in Figure 2, GalT activity in heterozygote GalT KO liver was significantly lower than in control liver, whereas α2,3 and α2,6ST activity was significantly higher than in controls. It is especially interesting that mRNA expression in heterozygote GalT KO liver mirror protein activity. As we expected, ELLA analysis showed that optical density of GS-IB4 for α-Gal epitope in heterozygote GalT KO liver (2.1138 ± 0.1864, $P < 0.001$) was significantly lower than that of the control (3.3282 ± 0.1775). However, there were significantly more Sia-containing glycoconjugate epitopes in GalT KO liver than in controls. Optical density of MAA (5.0459 ± 0.2541, $P < 0.05$) and SNA (25.8502 ± 1.0457, $P < 0.05$) in heterozygote GalT KO liver was significantly higher than those of the control (3.5984 ± 0.1192 and 20.0019 ± 2.2285, resp.; Table 2). These results suggest that α2,6ST may preferentially use Neu5Gc rather than Neu5Ac as a donor substrate so that heterozygote GalT KO pigs have more glycoconjugate-bound Neu5Gc epitopes than controls.

To examine whether increased production of Neu5Gc in heterozygote GalT KO pigs was caused directly by increased expression of CMAH, we tested whether the expression of full-length CMAH or variant mRNA in pig liver could be altered by deficiency of the GGTA1 gene. The full length of pig CMAH cDNA and its splicing isoform, variant 3, showed constant expression levels, whereas the CMAH variant 2 in heterozygote GalT KO livers was greater than that of controls (Figure 3). This suggests that CMAH variant 2 mRNA in heterozygote GalT KO liver may be involved in the underlying conversion mechanism from Neu5Ac to Neu5Gc.

Finally, we compared the proteomes of heterozygote GalT KO liver with control-derived liver by 2DE analysis (Figure 4). Heterozygote GalT KO livers showed a higher expression of NAD^+-isocitrate dehydrogenase (IDH), compared to those of controls (Table 3). IDH converts NAD^+ to

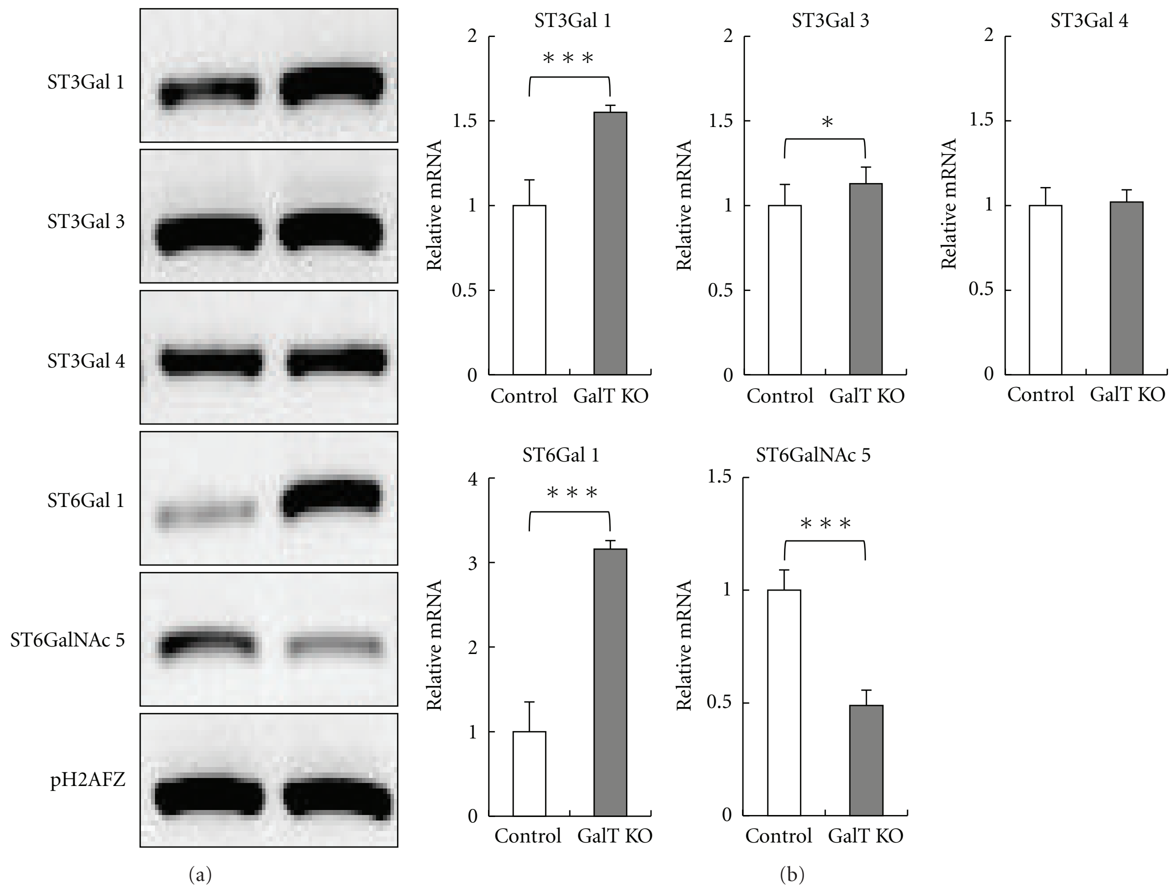

Figure 1: Expression of sialyltransferases in control and heterozygote α1,3-galactosyltransferase gene (GGTA1) knockout (GalT KO) liver. (a) Electrophoretic analysis of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in control and heterozygote GalT KO liver. (b) Quantification of real-time RT-PCR analysis in control and heterozygote GalT KO liver. All RT-PCR reactions were conducted in triplicate and normalized with pig H2A histone family, member Z gene (pH2AFZ). Each of the GalT KO relative values is presented as an n-fold expression difference compared to the control, which was set as 1. $^{*}P < 0.05$, $^{***}P < 0.001$.

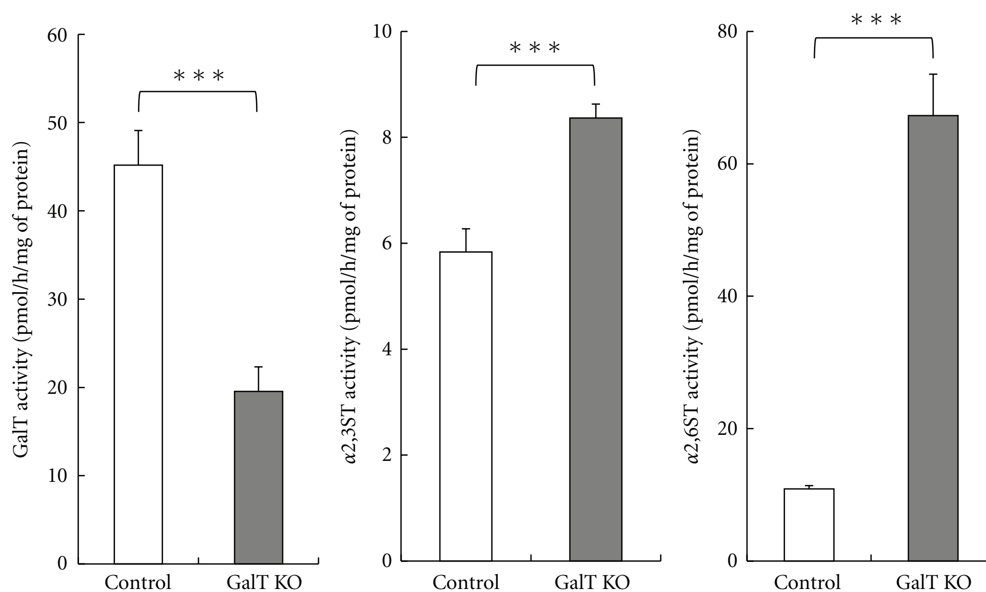

Figure 2: Comparison of α1,3-galactosyltransferase (GalT), α2,3- and α2,6-sialyltransferase (α2,3ST and α2,6ST) activity between control and heterozygote GalT KO liver. From left, GalT, α2,3ST, and α2,6ST activity. Each value is the mean ± standard deviation (SD) of triplicate determinations. $^{***}P < 0.001$.

Table 3: Upregulated proteins in heterozygote GalT KO liver.

SSP no.	Protein name	Score/SC (%)	pI/*M*r (kDa)	Accession no./data base
2109	Actin-capping protein beta chain, splice form 1	73/12	5.47/31.3	1083244/NC P79136/SP
2202	P1.11659_4	49/3	6.40/38.7	2984585/NC Q9UJZ1/SP
3005	Substrate protein of mitochondrial ATP-dependent proteinase SP-22	212/18	5.73/21.5	627764/NC P35705/SP
3202	NAD^+-isocitrate dehydrogenase, alpha subunit	135/11	5.72/36.7	1182011/NC P50213/SP
3221	NAD^+-isocitrate dehydrogenase, alpha subunit	99/11	5.72/36.7	1182011/NC P50213/SP
4103	Voltage-dependent anion channel 2	54/9	7.49/31.5	47523794/NC P68002/SP
4201	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42-kDa precursor	63/2	8.67/40.7	4758768/NC O95299/SP
6108	Endoplasmic reticulum protein 29 precursor	190/14	6.77/28.9	5803013/NC P30040/SP

SSP no. indicates the number of spots identified by PDQuest.
Score/SC indicates MASCOT score/Sequence coverage.

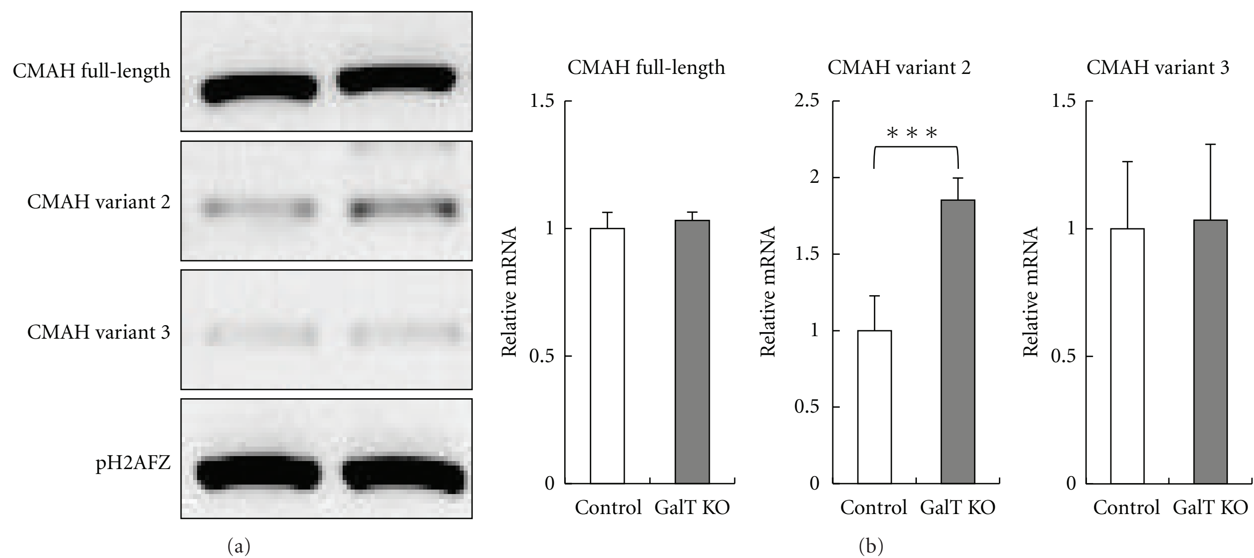

Figure 3: Expression of full-length CMAH and variants in control and heterozygote α1,3-galactosyltransferase gene (GGTA1) knockout (GalT KO) liver. (a) Electrophoretic analysis of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in control and heterozygote GalT KO liver. (b) Quantification of real-time RT-PCR analysis in control and heterozygote GalT KO liver. All RT-PCR reactions were conducted in triplicate and normalized for pig H2A histone family, member Z gene (pH2AFZ). Each of the GalT KO relative values is presented as an n-fold expression difference compared to the control, which was set as 1. $^{***}P < 0.001$.

NADH in the TCA cycle, and CMAH enzyme uses NADH as a cofactor for a hydrogen source in the catalytic reaction of CMP-Neu5Ac to CMP-Neu5Gc. These data suggest that Neu5Gc accumulation in heterozygote GalT KO pig-derived organs may be caused by an increase of α2,6ST and CMAH activity. The accumulation of Neu5Gc may result in progressive organ death.

4. Discussion

Sias are typically found at the terminal ends of oligosaccharide chains, which are involved in various biological processes, such as immune-response, inflammation, and tumor cell metastasis [17–19]. Neu5Ac and Neu5Gc are 2 of the most common Sias types. It is well-known that ST3Gal 1 and ST3Gal 4 catalyze the binding of α2,3-sialic acid

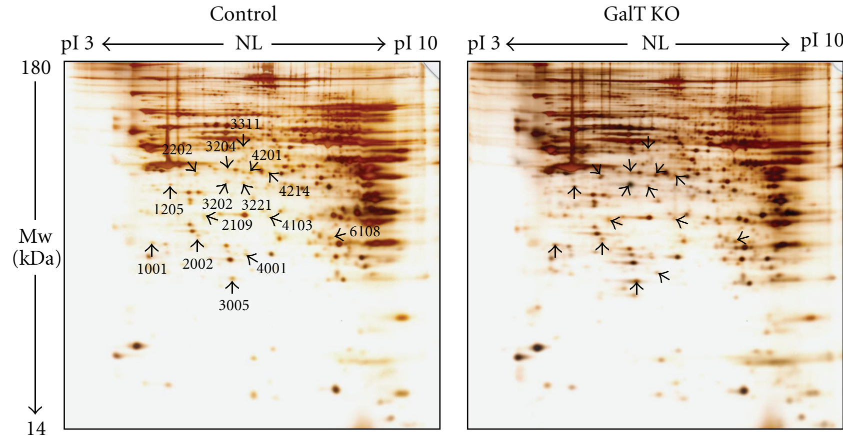

Figure 4: 2-D gel electrophoresis analysis of control and heterozygote GalT KO liver. Numbered spots were selected due to variations between the gels or overexpression in heterozygote GalT KO liver. Quantitative data were obtained from triplicate experiments. Identified spot numbers correspond to those in Table 3.

on Galβ1,3GalNAc-R to produce Siaα2,3Galβ1,3GalNAc-R, whereas ST3Gal 3 and ST6Gal 1 catalyze the binding of α2,3- and α2,6-sialic acid on Galβ1,4GlcNAc-R to produce Siaα2,3Galβ1,4GlcNAc-R and Siaα2,6Galβ1,4GlcNAc-R, respectively [16]. Additionally, ST6GalNAc 5 catalyzes the binding of α2,6-sialic acid on Siaα2,3Galβ1,3GalNAc-R to produce Siaα2,3Galβ1,3(Siaα2,6)GalNAc-R [16]. As shown in Figure 1, ST3Gal 1, ST3Gal 3, and ST6Gal 1 were upregulated in GalT KO pig liver cells as compared to controls. Similarly, both α2,3ST and α2,6ST were increased in heterozygote GalT KO livers compared to control livers. Therefore, upregulated ST3Gal 1, ST3Gal 3, and ST6Gal 1 may increase Siaα2,3Galβ1,3GalNAc-R, Siaα2,3Galβ1,4GlcNAc-R and Siaα2,6Galβ1,4GlcNAc-R, respectively, on glycolipid and glycoprotein within heterozygote GalT KO livers.

MAA consists of 2 molecular species, a hemagglutinating hemagglutinin (MAH) and a mitogenic leukoagglutinin (MAL). Both isolectins are able to interact with sialic-acid-contained glycoconjugates; MAH has higher affinity toward Siaα2,3Galβ1,3(Siaα2,6)GalNAc on *O*-glycan, but MAL preferentially binds to the Siaα2,3Galβ1,4GlcNAc structures of *N*-glycan chains [20, 21]. SNA lectin, however, specifically binds to Siaα2,6Gal/GalNAc structures of *N*- or *O*-glycan chains [22]. As expected, the signal level of MAA in heterozygote GalT KO liver was higher than that of the controls (Table 2). This result is reasonable because the decrease of GalT activity in heterozygote GalT KO liver, as compared to control livers, results in an increase in the nonreducing end (Galβ1,4GlcNAc-R) of glycan chains. The increased nonreducing ends allows the upregulated α2,3ST to easily produce Siaα2,3Galβ1,4GlcNAc-R. However, the SNA signal level in heterozygote GalT KO liver was similar to that of control liver. This may be explained by the decreased expression level of ST6GalNAc5 in the heterozygote GalT KO liver as compared to controls (Figure 1). The signal intensity of SNA toward Siaα2,6-containing glycoconjugates might be attenuated with a decrease in availability of Siaα2,6GalNAc. Shinkel et al. [23] reported that (1) GalT KO mice showed only a modest increase in *N*-acetyllactosamine residues and exhibited little sialylation and (2) Overexpression of H substance and suppression of the α-Gal epitope in HTF mice were associated with a marked reduction in α2,3-sialylation and exposure of normally cryptic antigens such as sialylated Tn and Forssman antigens. Pigs differ from mice, however, in that pigs have a 10- to 100-fold higher expression of α-Gal epitopes than mice have [24]. Additionally, GalT KO pigs showed up-regulation of sialylated epitopes compared to the nontransgenic wild type pigs [25]. It is not appropriate to compare lectin binding in pigs to lectin binding in mice because mice and pigs exhibit markedly different glycosyltransferase expression. In our study, GalT KO pigs showed up-regulation of α2,3- and α2,6-sialyltranferase compared to the control. Whereas mice splenocytes have cryptic epitopes in the inner cell, we used liver lysates in ELLA. All glycan epitopes of the organ are exposed within lysates and lectin (MAA and SNA) can directly bind to their sialylated epitopes. This study demonstrates that GalT KO pig has higher α2,3- and α2,6-sialylation when compared with those reported for GalT KO mice.

When CMP-Neu5Ac and CMP-Neu5Gc were compared as donor substrates, ST6Gal 1 showed 4–7 times greater activity toward CMP-Neu5Gc than CMP-Neu5Ac, whereas there was no significant difference between the activity of ST3Gal 1 toward these 2 substrates irrespective of the origin of the enzymes [26]. Similarly, the high level of ST6Gal 1 in heterozygote GalT KO liver may preferentially transfer Neu5Gc to the nonreducing galactose residue in glycan chains. These results indicate that a deficiency of GalT moderately increased α2,3-linked Neu5Gc glycoconjugates, but highly increased α2,6-linked Neu5Gc glycoconjugates.

NAD^+-related isocitrate dehydrogenase, also known as IDH, is an enzyme that participates in the citric acid cycle. It catalyzes the third step of the citric acid cycle, the oxidative decarboxylation of isocitrate, producing α-ketoglutarate and CO_2 while converting NAD^+ to NADH [27]. In order to produce Neu5Gc from Neu5Ac, CMAH requires cytochrome b_5 and NADH as cofactors [10, 11]. The heterozygote GalT KO liver showed up-regulation of NAD^+-related isocitrate

dehydrogenase alpha subunits, compared to control liver (Table 3). Recently, our group cloned the full pig CMAH cDNA [28]. The longest 1734 bp form encodes 577 amino acids and is designated as "full length". The shorter 1125 and 1056 bp forms designated as "variant 2" and "variant 3", have an in-frame stop codon and encode 374 and 351 amino acids, respectively. However, it remains unknown whether the CMAH-derived splicing isoforms have enzyme activity. As shown in Figure 3, the amount of variant 2 mRNA in GalT KO pigs was more significant than in controls, as determined by real-time RT-PCR. These observations suggest that CMAH variant 2 mRNA in heterozygote GalT KO liver may be involved in the underlying conversion mechanism from Neu5Ac to Neu5Gc. In the present study, we observed a clear decrease in GalT activity and increase in Neu5Gc content in heterozygote GalT KO pigs as compared to controls. In conclusion, Neu5Gc accumulation in heterozygote GalT KO pig-derived organs may be caused by a preference for Neu5Gc over Neu5Ac as a donor substrate due to upregulated α2,6ST and CMAH activity. Thus, the deletion of both the CMAH and GGTA1 genes in pigs is expected to further prolong xenograft survival. Even though heterozygote GalT KO pigs successfully produced fertilized sperm, the gnotobiotic facility in our system was limited. Therefore, while we were unable to acquire them, homozygote pigs might provide further clues to the long-standing question of why GalT KO-derived pig organs transplanted to baboon result in acute rejection at 179 days after transplantation.

Authors Contribution

J.-Y. Park and M.-R. Park equally contributed to this work.

Acknowledgment

This work was partially supported by Woo Jang-Choon (PJ007849) Projects from the RDA, Republic of Korea.

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